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Estimating recent growth in the cuttlefish *Sepia officinalis*: are nucleic acid-based indicators for growth and condition the method of choice?

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Abstract

A laboratory calibration study was undertaken with juvenile *Sepia officinalis* (80–85 g initial wet weight) to investigate the effects of different food rations and different starving intervals on RNA/dry weight (DW) ratios and RNA/DNA ratios in cephalopod mantle muscle at two different temperatures. The digestive gland index was also used as an additional indicator of recent growth. High food rations and low temperature went along with high RNA/DW ratios and high RNA/DNA ratios. Starving resulted in a linear decline in growth performance and a concomitant decrease in RNA/DW and RNA/DNA ratio, with RNA/DNA ratios representing the growth data better. RNA/DNA ratios decreased faster at higher temperatures. A fluorimetric assay for nucleic acid analysis was optimized for cephalopod mantle tissues and yielded reproducible RNA/DNA ratios with a relative variance below 10%. Thus, it may be possible to use this estimator of recently encountered feeding regime for the evaluation of mortality rates of early teuthid paralarvae to eventually support stock management. Also, log relative digestive gland weight showed a strong relationship with starving time, but, surprisingly, not with temperature. Data from the two temperatures analyzed could be combined to form a common regression line of relative digestive gland index with starving time. This indicator for recent growth might be especially suitable for large specimens with a well-developed digestive gland.

Keywords: RNA/DNA; *Sepia officinalis*; Growth

1. Introduction

Estimation of the nutritional status of commercially exploited marine organisms is an important task in the field of fisheries biology. Especially in recruitment studies, predictions on early life mortality are crucial

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to estimate future stock biomass. For a wide variety of fish species, such studies have already been performed successfully using nucleic acid-based indicators for growth and condition (Clemmesen, 1989, 1992, 1994, 1996; Bergeron, 1997; Buckley et al., 1999; Moksness et al., 2000, Clemmesen et al., 2003) and common standards have been established (Caldarone et al., 2001). Cephalopod tissue has also been analysed for nucleic acid content (Clarke et al., 1989; Houlihan et al., 1990a,b, 1998; Castro and Lee, 1994; Pierce et al., 1999; Koueta et al., 2000; Vidal, 2000), but results are hard to compare, since a variety of different methods have been used. Some of these studies have met limited success in relating RNA/DNA ratios to growth rates (Clarke et al., 1989); others find good relationships between these parameters (Castro and Lee, 1994). Houlihan et al. (1998) used RNA/protein ratios as an index for recent growth in *Eledone cirrhosa*, while Pierce et al. (1999) suggested the use of RNA concentrations for such purposes, arguing that systematic variation in protein concentration would undermine the RNA/protein index. RNA/DNA values determined so far for cephalopod species range between 1 and 50, depending on the particular method used. Thus, the need for a common standard exists to make results between research groups more comparable. This standard assay needs, not only to be reliable and sensitive, but also to be reproducible, inexpensive, and fast. Sensitive fluorimetric assays that can be carried out by multititer plate readers, as currently employed by Clemmesen et al. (2003) and Caldarone et al. (2001) for larval fish samples, seem to be the best alternative to rapidly analyze large quantities of samples. The present study implements such an assay on cephalopod tissue samples by trying to answer the following questions:

(1) Can RNA/DNA ratios or RNA/dry weight (DW) ratios ([RNA]) serve as accurate indices for recent growth in a cephalopod species?
(2) How do these indices compare to a somatically derived index like the digestive gland index (DGI)?
(3) Can these indices predict the duration of encountered starving regimes?
(4) Does temperature as the main abiotic environmental factor affecting marine ectothermic animals have an effect on these indices?

To clarify these questions, a laboratory calibration experiment was designed with late juvenile *Sepia officinalis* as the model cephalopod organism. All environmental factors were controlled and individuals kept separate to solely consider the effects of food availability and temperature on the different indices for recent growth estimation.

2. Materials and methods

2.1. Culturing systems

All experiments were performed in floating cages made of a PVC framework, which was covered with nylon netting (mesh width 2 mm). One PVC frame contained five compartments, each enclosing a volume of 13,800 cm^3 (30×20 cm, 23 cm deep).

In order to perform experiments at two different temperatures, two different seawater culture systems adjacent to one another were used. Tank system A was a raceway system with a total water volume of 5000 l. This system was chosen to be heated 5 °C above the temperature of system B. Tank system B consisted of a circular tank with a total water volume of approximately 50,000 l and filtration units. Both tank systems were closed recirculating seawater systems using natural seawater. No water changes were made during the experiments.

Although slightly different in design, both culture systems utilized the same sequential water filtration processes to maintain high water quality. Water leaving the animals in culture was first passed through layers of polyester batting material to remove suspended particulates. Next, the water passed through an upwelling activated carbon contacting chamber to remove dissolved organics and finally through large crushed oyster shell biological filter beds. Neither protein skimming nor UV disinfection was employed in these systems during these experiments. Similar systems have been previously described in greater detail (Yang et al., 1989; Hanlon, 1990; Forsythe et al., 1994). Water transport was achieved by electric centrifugal pump in tank system A and by a large volume airlift pump in tank system B. Food remains in the tanks were removed daily. Water quality parameters (concentrations of ammonia, nitrite, and nitrate) were assessed twice a week, employing standard spectrophotometric

tests (Hach, Loveland, Colorado, USA). Temperature and salinity were monitored twice a day (09:00 and 20:30 h). The pH was maintained above 7.8, with regular additions of sodium bicarbonate solution when pH decreased. No trace elements were added during the experiments. Light was provided by overhead fluorescent bulbs from 8:30 h until 20:30 h everyday.

2.2. Experimental animals

The animals used in the present experiments were selected based on similar body wet weight from a large culture population at the National Resource Center for Cephalopods (NRCC) at Galveston, TX. These animals were the F1 generation of a culturing stock, which originated in egg masses brought from the French Atlantic coast.

Within the experimental cages, the animals were habituated to the feeding situation and the new surroundings for 4 days. Experiments were only started when all animals readily accepted the food offered. The exact wet weight of each specimen was taken on the first day of the experiment. Animals were taken out of the water and held at a downward angle for approximately 20–30 s. During this time period, animals violently expelled remaining water from the mantle cavity and could be weighed on a Sartorius precision scale. Prior experimentation with magnesium chloride anaesthesization (Messenger et al., 1985) to relax animals before weighing was unsuccessful because animals appeared to be stressed by the procedure, resulting in chronic inking and escape jetting behavior within the narrow cages afterwards, which subsequently led to skin lesions.

2.3. Experimental design

Two experiments were designed to test the effects of temperature and food availability on growth, RNA and DNA concentrations, and relative digestive gland weight. Experiment 1 tested the effects of two different feeding rations at two temperatures; experiment 2 tested the effects of different starving intervals in relation to temperature.

In order to design different feeding groups, a 24-h maximum ingestion experiment was performed with the experimental animals. All experiment 1 animals were fed ad libitum with frozen shrimp (*Penaeus aztecus*). The fresh weight consumed was recorded for each animal, a mean maximum daily ingestion rate (MIR) calculated for both temperatures, which served as the basis for later calculation of individual feeding rations in the two experiments:

$$MIR[\%\mathrm{BW/d}] \text{ at } T_{23^\circ\mathrm{C}/18^\circ\mathrm{C}} = \frac{1}{n}\sum_{i=1}^{n}\frac{\mathrm{FW(ing.)}_{xi}[\mathrm{g}]}{t[\mathrm{d}]*\mathrm{BW}_{xi}[\mathrm{g}]} \times 100$$

where MIR [%BW/day]=mean maximum daily ingestion rate in percent of mean initial bodyweight of experimental animals; $\mathrm{FW(ing)}_{xi}$ [g]/1 [days]=fresh weight in grams of *P. aztecus* ingested in 24 h by experimental animal$_{xi}$; BW_{xi} [g]=bodyweight of experimental animal$_{xi}$ at start of experiment; n=number of experimental animals (20 per temperature).

MIRs were 24% (standard deviation (S.D.)=3.8%) of their own bodyweight per day (BW/day) for *S. officinalis* at 23 °C and 12% BW/day (S.D.=4.5%) at 18 °C.

In experiment 1, four treatment groups were designed, two at each temperature. At each temperature, one group of animals (n=10) received a low ration (LF) and one (n=10) a higher ration (HF). To make low ration and higher ration groups between temperatures statistically better comparable, they received the same percentage of their temperature-dependent mean maximum ingestion rate. Thus, the low ration group at 18 °C and the low ration group at 23 °C were fed 16.67% of their MIR, which corresponds to 4% of their own body weight per day of food at 23 °C and 2% of their own body weight per day of food at 18 °C; the high ration animals received 50% of their MIR, which corresponds to 12% BW/day at 23 °C and 6% BW/day at 18°C. The duration of this experiment was 10 days (see Table 1).

In experiment 2, four groups of animals at 17.5 °C and four at 22.5 °C were fed a constant 50% MIR and afterwards starved for defined time intervals of 0, 2, 4, or 6 days (see Table 1). This experiment lasted 7 days.

Experimental animals were kept separately from one another; cages were assigned to animals from different groups randomly. Frozen shrimps (*P. aztecus*) were thawed every morning and cut into pieces according to assigned feeding group and initial weight of each cephalopod (i.e., an animal initially weighing 90 g, assigned to group 2 in experiment 1 at 23 °C

Table 1
Experimental design and animal body weights

Experiment number/group	n	W_1 (S.D.) [g]	W_2 (S.D.) [g]	Δt [days]	Feeding ration [%BW/day]	[%MIR]
Experiment 1						
LF, 18 °C	10	82.9 (9.0)	92.8 (8.9)	10	2	16.7
HF, 18 °C	9	82.5 (8.5)	102.0 (9.2)	10	6	50
LF, 23 °C	10	84.5 (7.3)	98.2 (8.7)	10	4	16.7
HF, 23 °C	10	84.3 (6.7)	125.8 (10.2)	10	12	50
Experiment 2						
0 days of starvation, 17.5 °C	5	84.2 (7.7)	103.1 (12.3)	7	6	50
2 days of starvation, 17.5 °C	5	84.0 (8.9)	97.1 (9.9)	7	6	50
4 days of starvation, 17.5 °C	5	84.6 (6.8)	92.2 (9.4)	7	6	50
6 days of starvation, 17.5 °C	5	84.3 (7.7)	88.3 (8.4)	7	6	50
0 days of starvation 22.5 °C	5	85.3 (8.3)	116.3 (14.3)	7	12	50
2 days of starvation, 22.5 °C	5	82.6 (9.6)	103.4 (10.4)	7	12	50
4 days of starvation, 22.5 °C	5	83.6 (9.1)	98.6 (11.3)	7	12	50
6 days of starvation, 22.5 °C	5	83.3 (8.9)	86.4 (9.8)	7	12	50

LF=low ration groups that were fed 16.7% of their MIR daily; HF=high ration groups that were fed 50% of their MIR daily; W_1=mean body mass at the start of the experiment; W_2=mean body weight at the end of the experiment; S.D.=standard deviation; Δt=period between W_1 and W_2 in days.
Feeding ration was expressed as (a) percent of maximum ingestion rate/day at the respective temperature (MIR) and (b) percent of initial bodyweight/day.
Experiment 2: animals were fed 50% MIR daily until the start of the defined starving period.

given an imposed feeding rate of 50% MIR/day, which corresponds to 12% BW/day, would receive 0.12×90 g=10.8 g of shrimp meat each day) and were fed individually. The slight differences in experimental temperatures between the two experiments (which were performed consecutively) were due to problems with the air-conditioning unit that controlled the water temperature during the second experiment.

Feeding times were 09:00 h (experiment 1) and 12:00 h (experiment 2). As animals usually did not ingest all offered foods instantly, food remains were removed and weighed the following day within 30 min before the next feeding event.

Experiments were terminated by anaesthesizing the cuttlefishes in cold (0 °C) seawater for 4 min, then decapitating the animals with a scalpel. Weight was recorded, then mantle tissue samples were taken and immediately deep-frozen at −70 °C. Animals were dissected afterwards, sex was determined, and digestive glands were removed and weighed.

Tissue samples for biochemical measurements were transferred to Germany on dry ice and stored at −74 °C for 2 months prior to analysis.

Mantle samples for biochemical analysis were taken as dorso-ventral transects at a fictional line on the animals' (physiological) ventral side, which would connect right and left body sides at about 50% mantle length. Pieces of 50–200 mg fresh weight were taken. This procedure was found to be necessary, since different portions of the muscle mass may be characterized by a different nucleic acid composition, as has been shown for fish species (Caldarone, unpublished, in Buckley et al., 1999).

2.4. Biochemical analysis

Nucleic acid concentrations were determined using a modified fluorescent dye-based method (Clemmesen et al., 2003; Belchier et al., 2004). Ethidium bromide (EB; 3,8-diamino-6-phenyl-5-ethylphenantridium bromide; SERVA 31238) was used as fluorophore. This molecule binds by intercalation and therefore is specific to double-stranded polynucleotides (DNA). For single-stranded polynucleotides (RNA), secondary and tertiary structures will determine the amount of EB that will be able to intercalate. Nucleic acid fluorescence is enhanced by a factor of 20–30 through the binding of EB, when being excited at 355 nm (emission peak at 590 nm) (Le Pecq and Paoletti, 1966; Le Pecq, 1971).

Mantle tissue samples from the experimental animals were freeze-dried until weight constancy (16

h), then weighed with a Sartorius balance to the nearest 0.1 mg. Homogenization of the tissue was performed in distilled water in 10 ml plastic vials. Distilled water was added to reach a final concentration of 8 mg dry weight/ml water. Samples then were homogenized for 30 s with an Ultraturrax homogenisator, followed by 15 s with a Sonifer cell disrupter and an additional 15 s with the Ultraturrax. 100 μl of the crude homogenate was transferred to 1.5 ml Eppendorf caps filled with 300 μl of 0.05 M Tris–EDTA buffer with a sodium dodecyl sulphate (SDS) concentration of 0.05%. Constant amounts of glass beads (2 mm and 0.2 mm diameter) were added and the caps treated in a Retsch MM2 shaking mill for 15 min at maximum power. After centrifugation (Heraeus Minifuge T; 8 min, 3800 g, 0–4 °C), 280 μl of the supernatant was transferred to new caps and used in the assay. All homogenization and preparatory steps were performed on ice.

Samples were placed on black 96-well microtiter plates (LabSystems *Cliniplate*). Since EB was used for both determination of RNA and DNA, 100 μl of each sample was used for the determination of total fluorescence, while another 100 μl was treated with RNAse (Ribonuclease A, bovine pancreas, SERVA 34388; 25 μl was added to each well) in order to only assess the proportion of total fluorescence caused by EB intercalating with DNA. Thus, RNA concentration could be backcalculated (Clemmesen, 1994; Clemmesen et al., 2003; Belchier et al., 2004).

All following steps were performed by a microtiter reader with dispensing functions (LabSystems, *Fluoroscan Ascent*), controlled by a PC. 180 μl or 155 μl of Tris–EDTA buffer without SDS, respectively (a difference of 25 μl to compensate for the volume of the RNAse fraction in the DNA samples), was automatically added by dispensers to dilute the homogenate. After recording of self-fluorescence of all samples, 20 μl of EB was added to the wells without RNAse (to measure total fluorescence). DNA fluorescence wells were incubated for 30 min at 37 °C to promote digestion of RNA, left to cool for 30 min, whereafter 20 μl of EB was added. Fluorescence was recorded 130 min after EB incubation.

Standard curves were established for RNA (ribosomal 16 s, 23 s from *Escherichia coli*; Boehringer Mannheim GmbH 206938) and DNA (phage λ-DNA; Boeringer Ingelheim). RNA and DNA concentrations derived from these after subtraction of sample self-fluorescence and EB self-fluorescence. DNA standard curves were prepared once-a-week RNA standard curves with every plate read. Dilution series of RNA and DNA standards were measured in the same buffer concentrations as the samples. Each curve consisted of five data points, with each point of two replicates.

From each homogenate, three samples were taken and measured, and mean values for nucleic acid concentrations were taken.

2.5. Calculations

Growth was expressed as instantaneous relative growth (G) (Ricker, 1979; Forsythe and van Heukelem, 1987):

$$G = 100^{*}\frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

where W_2=final fresh weight of experimental animal at the end of the experiment (in g); W_1=initial weight of experimental animal (in g); and (t_2-t_1)=time interval between weight measurements (in days).

2.6. Indices for recent growth estimation

Biochemical indices examined were RNA concentration (=[RNA]) (expressed as μg RNA/mg mantle muscle dry weight (DW)) and RNA/DNA ratios (expressed as μg RNA/mg mantle muscle DW divided by μg DNA/mg mantle muscle DW).

Table 2
Experiment 1 two-factorial ANOVA results

Factors	RNA/DNA	p	[RNA]	p	DGI	p	G	p
Temperature	$F_{(1,34)}$=17.67	<0.001	$F_{(1,34)}$=15.85	<0.001	$F_{(1,35)}$=4.98	<0.04	$F_{(1,35)}$=73.5	<0.001
Ration	$F_{(1,34)}$=33.62	<0.001	$F_{(1,34)}$=17.52	<0.001	$F_{(1,35)}$=407.2	<0.001	$F_{(1,35)}$=177.6	<0.001
Temperature×ration	$F_{(1,34)}$=7.28	<0.02	$F_{(1,34)}$=2.37	<0.14	$F_{(1,35)}$=0.03	<0.86	$F_{(1,35)}$=33.8	<0.001

A digestive gland index (DGI) was constructed by dividing digestive gland fresh weight by whole animal fresh weight (including the digestive gland organ). Other authors (Pierce et al., 1999) excluded gonad weight from total body weight, expressing digestive gland weight as a proportion of somatic body weight.

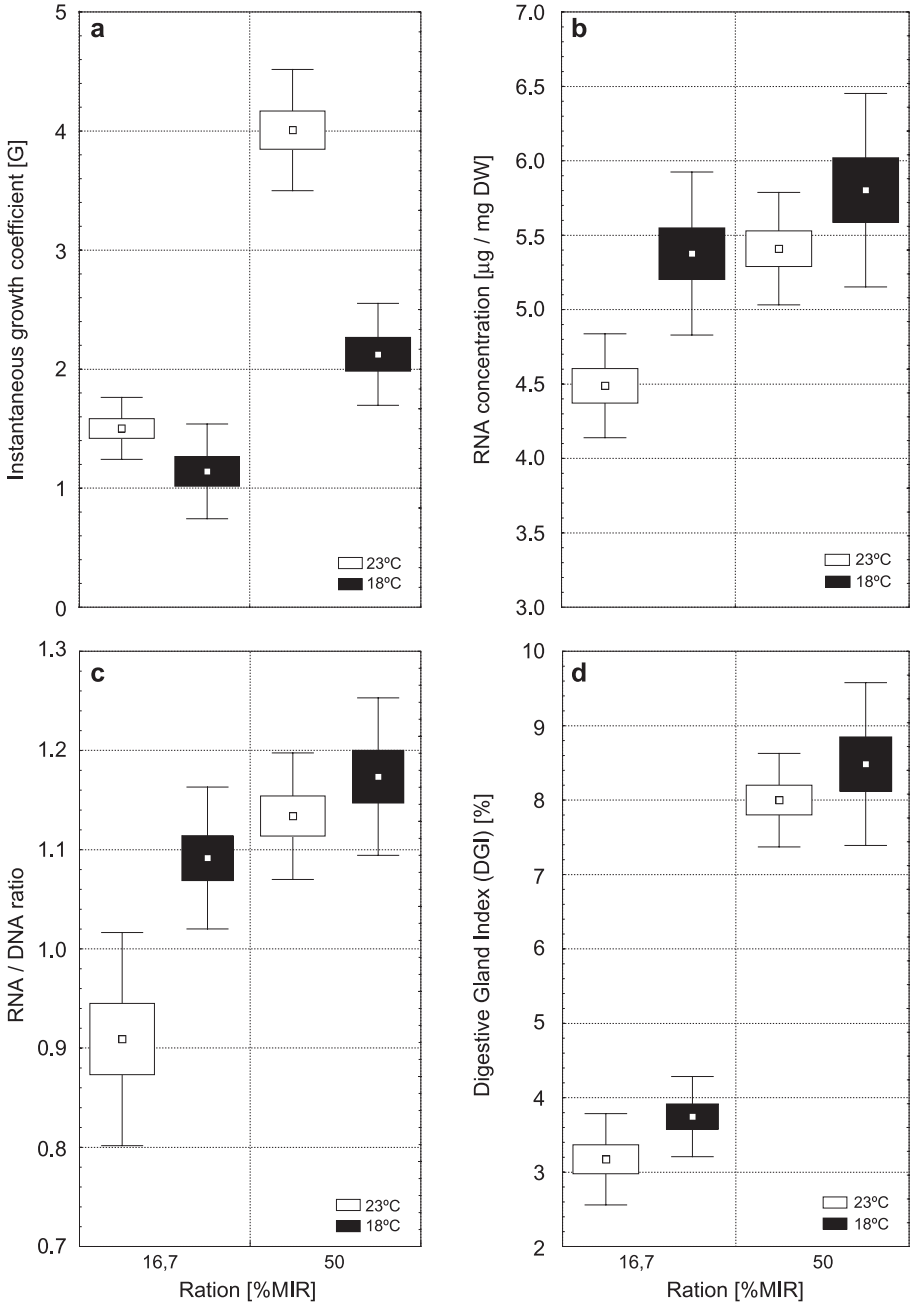

Fig. 1. Experiment 1. Boxplots (mean–S.E.–S.D.). (a) Instantaneous growth coefficient (*G*) vs. feeding ration. (b) RNA concentration vs. feeding ration. (c) RNA/DNA ratio vs. feeding ration. (d) Digestive gland index (DGI) vs. feeding ration. Biochemical indices derived from mantle muscle samples.

Since all experimental animals in this study were juveniles and of approximately the same weight, this study proceeded as indicated above.

2.7. Statistics

Statistical analysis were performed using STATISTICA software (Statsoft, Version 5.1, 1997).

Experiment 1 results were analyzed using a two-factorial ANOVA (factors: temperature and feeding ration). Simple linear regression analysis was performed with experiment 2 results. Selected regression curves from different temperatures in experiment 2 were compared by testing for heterogeneity of slopes. Exponential curves were log-transformed to conduct regression analysis, tests for heterogeneity of slopes, and ANCOVA.

3. Results

3.1. General

Water quality parameters did not exceed the values recommended for the culture of marine animals in general (Spotte, 1979) or cephalopod culture (Hanlon, 1990). No animal losses were encountered during the experimental course.

Between 88% and 100% of the food offered was ingested by all experimental animals, enabling us to statistically compare between-treatment factors. A parallel trial to experiment 1 running at 83% MIR showed that animals were not able to ingest such an amount of food daily. The growth rates of this group could not be distinguished from the growth rates of the 50% MIR groups; thus, one can anticipate that the 50% MIR rate applied in a 10-day-long experiment actually represents the highest continuous daily food intake *S. officinalis* specimens within this particular size range can manage to ingest.

3.2. Growth

In experiment 1, animals at 23 °C displayed mean G values of 1.5 at the lower feeding ration (LF) and 4.01 at the higher feeding ration (HF), increasing in mean body weight from 84.5 g to 98.22 g at LF and from 84.26 g to 125.82 g at HF. At the lower temperature (18 °C), LF animals displayed G values of 1.14; HF animals grew at a G of 2.12. This corresponded to weight increases from 82.92 g to 92.84 g at LF and from 82.53 g to 101.96 g at HF. Animal weights of all four groups in experiment 1 could be considered equal at t=0 and differed significantly after 10 days of experimental treatment. Two-factorial ANOVA (factors: temperature and feeding ration) revealed a significant effect of temperature (p<0.001), feeding ration (p<0.001), and interaction between factors (p<0.001) on attained growth rates. (Tables 1 and 2, Fig. 1).

In experiment 2, animals from all eight treatment groups could be considered equal in terms of body weight at the beginning of the experiment. Table 1 lists the initial and final weights of these animals. Regression analysis (factor: starving time; dependent variable: G) at 17.5 and 22.5 °C yielded significant results (p<0.001 for both regressions; Table 3, Fig. 2). A test for heterogeneity of slopes produced a significant result (p<0.001); thus, it can be stated that growth rates decreased faster at the higher temperature under starving conditions.

3.3. Nucleic acid measurement protocol

Preliminary trials suggested that a higher detergent (SDS) concentration should be used than for fish tissue assays currently used in our laboratory (e.g., Malzahn et al., 2003; Belchier et al., 2004). Higher SDS concentrations led to lower RNA/DNA values because more DNA was extracted from the tissue. The lowest relative variances between replicates of the same mantle homogenate could be attained by using 0.05% SDS in the extraction buffer fraction. Higher

Table 3
Regression analysis of experiment 2 results

Index	T [°C]	Regression equation	R^2	p
G	17.5	$Y=-0.26x+1.97$	0.89	<0.001
G	22.5	$Y=-0.44x+3.15$	0.78	<0.001
RNA/DW	17.5	$Y=-0.11x+5.33$	0.26	<0.03
RNA/DW	22.5	$Y=-0.29x+5.66$	0.56	<0.001
RNA/DNA	17.5	$Y=-0.024x+1.074$	0.51	<0.001
RNA/DNA	22.5	$Y=-0.043x+1.09$	0.74	<0.001
Log DGI	17.5	$Y=-0.98x+0.86$	0.94	<0.001
Log DGI	22.5	$Y=-0.106x+0.88$	0.96	<0.001
Log DGI	Combined	$Y=-0.99x+0.876$	0.88	<0.001

x=starving time [days].

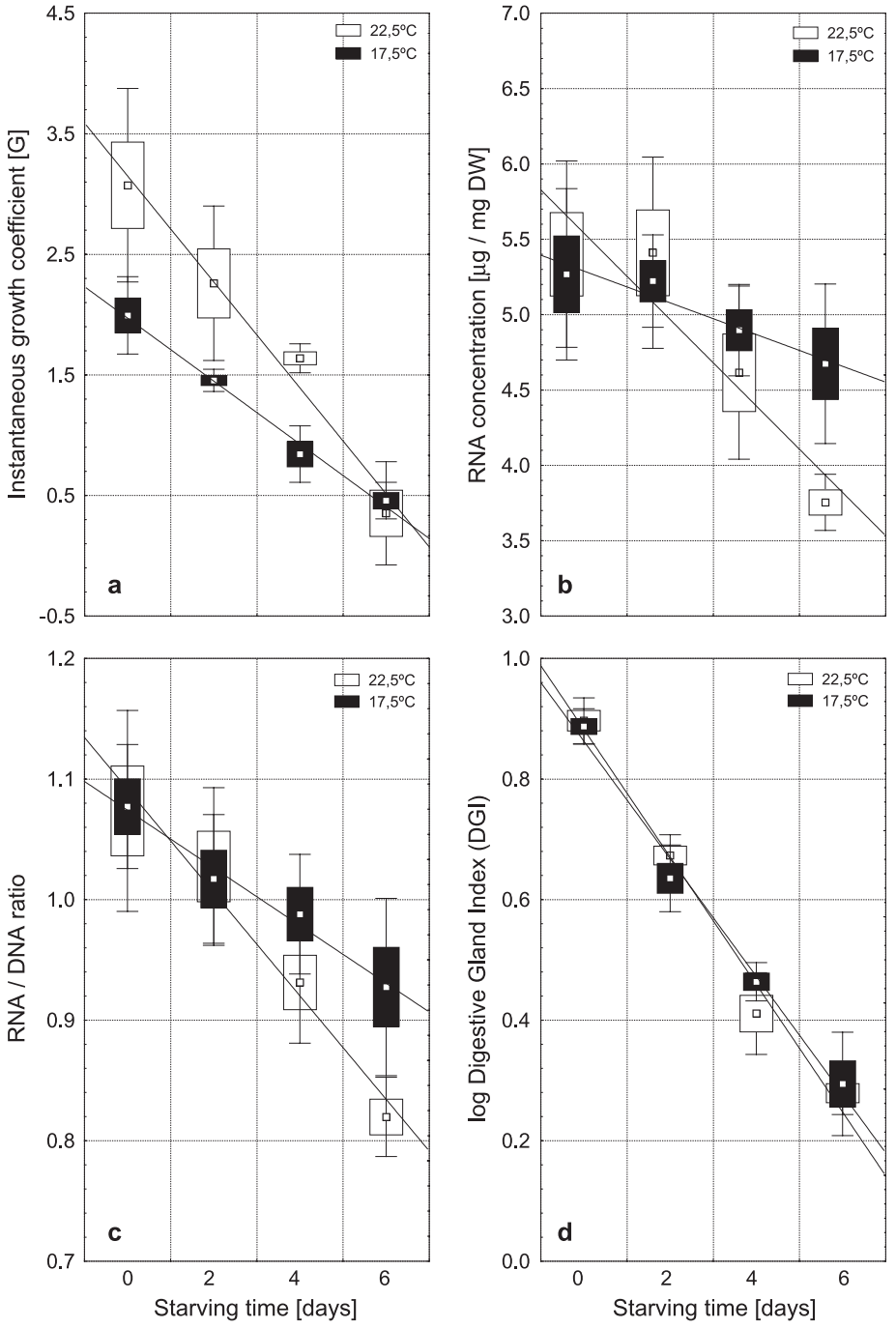

Fig. 2. Experiment 2. Boxplots (mean–S.E.–S.D.). (a) Instantaneous growth coefficient (G) vs. starving time. (b) RNA concentration vs. starving time. (c) RNA/DNA ratio vs. starving time. (d) Digestive gland index (DGI) vs. starving time. Biochemical indices derived from mantle muscle samples. Linear regression lines added; see Table 3 for regression analysis.

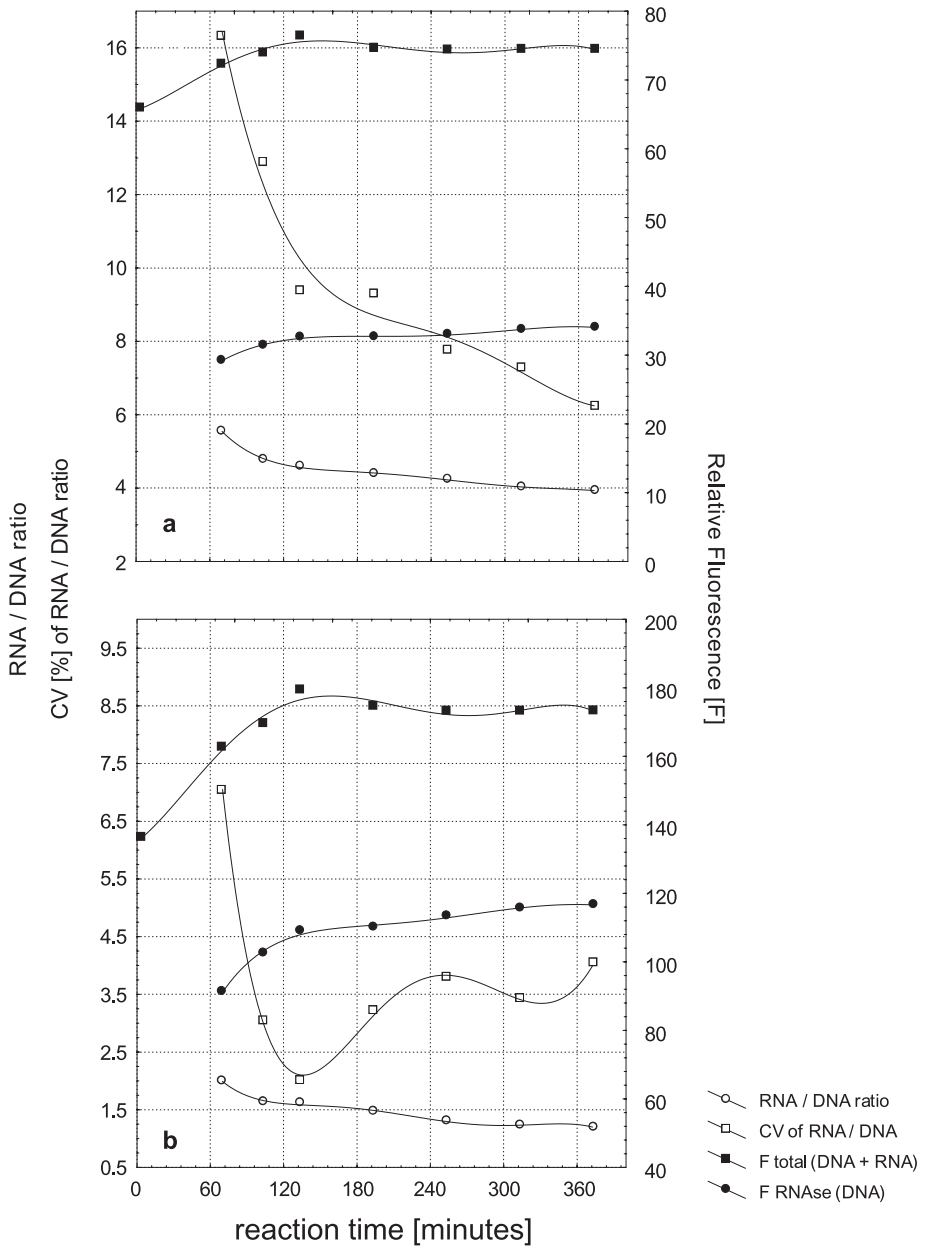

Fig. 3. (a) Effects of 0.01% SDS in the extraction buffer fraction. Fluorescence values were followed over time; resulting RNA/DNA ratios and CV (=relative standard deviation in percent) of mean RNA/DNA ratios were added to the graph. Fifth-order polynomials were fitted to the graphs. Data points represent eight replicate samples from one mantle tissue homogenate from one specimen. F_{total} (DNA+RNA)=total fluorescence samples (caused by EB intercalating with the homogenate's RNA and DNA). F_{RNAse}=DNA fluorescence (caused by EB intercalating with DNA after RNA has been digested by RNAse). Reaction time=0 represents the point of time at which EB is added to the RNA+DNA samples (=total fluorescence samples). Between this point and the measurement point at approximately 70 min lie the incubation of DNA samples with RNAse (30 min) and a cooling period (30 min) followed by the addition of EB to these samples. Thus, the first data point of F_{RNAse} (=[DNA]) appears at t=70 min. This same trial was repeated twice with mantle tissue homogenates from two other, randomly chosen specimens, yielding similar results. (b) Effects of 0.05% SDS in the extraction buffer fraction. This graph shows another set of eight replicate samples from the same crude homogenate that was used for (a). This time 0.05% SDS was used in the extraction buffer fraction. Note the different y-axis scaling.

and lower concentrations led to higher relative variances. The fluorimetric assay used in the present study was also found to be relatively sensitive to EB incubation time and homogenate concentration (mg DW tissue/ml distilled water). Variation in each of these factors could alter the measured RNA/DNA ratio, possibly masking subtle treatment effects.

Fig. 3a and b display some of these effects: one *S. officinalis* mantle muscle homogenate was divided into 16 subsamples of which eight were treated with 0.01% (Fig. 3a) SDS extraction buffer and eight with 0.05% (Fig. 3b). All samples were treated according to the described assay protocol, but fluorescence values were followed for a time period of approximately 370 min. Derived RNA/DNA ratios and relative variances of RNA/DNA ratios were calculated. Using the lower detergent concentration produced RNA/DNA values more than twice as high as with the higher concentration, while, on the other hand, relative variance of RNA/DNA ratios was higher at the lower concentration, indicating lower and less stable nucleic acid extraction rates.

3.4. RNA/DW ratios ([RNA])

Significant differences in [RNA] between LF and HF groups in experiment 1 ($p<0.001$) could be shown, with higher [RNA] found within HF samples. Significant differences in [RNA] between the two temperatures were also observed ($p<0.001$). Low temperature went along with higher [RNA]. No significant effects between factors on [RNA] were observed ($p>0.13$) (Fig. 1, Table 2).

RNA/DW ratios could be shown to linearly decline with starving time at both temperatures (Fig. 2). Regression analysis revealed that slopes at 22.5 °C ($p<0.001$) and 17.5 °C ($p<0.03$) were significantly different from zero. Linearity could be assumed in both cases ($p>0.89$ at 22.5 °C; $p>0.92$ at 17.5 °C) (Table 3).

3.5. RNA/DNA ratios

Significant differences in mantle RNA/DNA ratios between the two feeding rations were observed ($p<0.001$). Higher RNA/DNA ratios were found within HF animal mantle muscle tissues. Temperature also had a significant effect on the height of the ratios ($p<0.001$), with higher ratios found at the lower temperature. Significant effects between factors were also encountered ($p<0.01$) (Fig. 1, Table 2).

In experiment 2, analysis of regression was applied to data from both temperatures, yielding insignificant results for both. Linearity could be assumed in both cases and slopes differed from zero ($p<0.001$ at both temperatures) (Fig. 2). A test for heterogeneity of slopes ($p<0.03$) revealed differences in slope between the two temperatures with the higher negative slope at the higher temperature (Table 3).

3.6. Digestive gland index

Digestive gland index (DGI) ranged between 2.4% (2.9%) BW and 9.4% (10.6%) BW in experiment 1 at 23 °C (18 °C), and between 1.7% (1.8%) BW and 8.4% (8.5%) BW in experiment 2 at 22.5 °C (17.5 °C), demonstrating a great flexibility of the digestive gland organ in response to feeding conditions.

In experiment 1, two-factorial ANOVA revealed that temperature had a significant effect on the height of the digestive gland index ($p<0.04$). Higher DGI values were found at the lower temperature. Ration had an even higher impact on DGI values ($p<0.001$): higher food rations went along with higher DGI values ($p<0.001$). No significant effects between factors could be observed ($p>0.85$) (Fig. 1, Table 2).

Starving time also affected the DGI. A strong decline during the first four starving days and slower decline thereafter could be seen. Very similar exponential fits best represent the data at both temperatures (Fig. 2). A test for heterogeneity of slopes of log-transformed DGI values from both temperatures proved to be nonsignificant ($F_{1,36}$=0,11; $p>0.74$). Analysis of covariance of log-transformed DGI data yielded a nonsignificant result ($F_{1,37}$=0,28; $p>0.59$). Subsequently, data from starving groups at both temperatures were pooled and a common regression line was constructed (Table 3).

4. Discussion

4.1. Analytical methods

The assay used proved reliable for the determination of nucleic acid concentrations in cephalopod

tissue, although it could be demonstrated that the choice of detergent concentration in the extraction buffer fraction greatly influenced the stability of nucleic acid extraction and the height of attained RNA/DNA ratios. This was largely due to higher and more stable DNA yields at the higher SDS concentration.

Studies that employ assays, which work without detergents (Vidal, 2000; *Loligo opalescens* whole animal homogenates) or lower concentrations of detergents (Clarke et al., 1989; *S. officinalis* whole animal homogenates) in the extraction buffer, produced higher RNA/DNA values and higher relative variances. This probably reflects low nucleic acid extraction rates. Clarke et al. (1989), using 0.01% SDS, could not correlate DNA concentrations of whole animal extracts with animal wet mass, indicating incomplete and unstable DNA extraction rates.

These findings suggest that for every new species or taxa to be analyzed, optimum conditions for nucleic acid extraction should be determined in order to produce reliable results. Assay conditions should not be allowed to vary between samples. Helpful in this regard is the use of automated systems that perform critical steps in the assay with great precision and reproducibility (incubation time intervals at fixed temperatures, dispensing of EB, and standardized shaking procedures). Valuable suggestions for an assay evaluation protocol have been made by Buckley et al. (1999). Comparing the height of attained RNA/DNA ratios between different species (e.g., Frantzis et al., 1992) will only reveal significant results given that intercalibration exercises are undertaken.

4.2. Growth

Maximum growth rates attained in the present study (Figs. 1 and 2) are comparable to those of previous ad libitum feeding laboratory growth studies (Castro and Lee, 1994; DeRusha et al., 1989; Domingues et al., 2001; Table 4). Although small *S. officinalis* (<10 g) can grow rapidly at G values of >8 (Forsythe et al., 2002), animals >100 g could not be shown to display G values >3. Thus, our maximum G values of 3–4 at the higher temperature and 50% MIR actually seem to represent the highest possible growth rates feasible under the respective laboratory conditions. Correspondingly, animals kept at 83% MIR were not able to ingest all offered foods and their growth rates could not be distinguished from those of the 50% MIR group. Growth rates of wild animals of comparable weight as derived from fisheries data (Table 4) did not exceed those attained in the mentioned laboratory studies.

Table 4
Cuttlefish maximum growth rates

Study	Mass range [g]	Temperature [mean]	G	Laboratory-reared
1	106–170	21	2.8	+
2	30–209	22	2.9	+
3	110–126	27	1.31	+
4 (Males)	95–234	?	3.02	–
4 (Females)	156–245	?	1.51	–
5	2–24	25	8.4	+
5	2–5	17	3.5	+

(1) Castro and Lee (1994), animals fed frozen shrimp ad libitum for 17 days; (2) DeRusha et al. (1989), animals fed frozen shrimp ad libitum for 2 months; (3) Domingues et al. (2001), animals fed ad libitum with frozen crabs (*Carcinus maenas*), growth rate of the last 10-day period of their experiment 1; (4) Dunn (1999), growth rates calculated from length–frequency data and respective length–weight relationships of cuttlefish catches from the English Channel between July and August 1994; (5) Forsythe et al. (2002), animals fed ad libitum with live mysids.

4.3. [RNA] and RNA/DNA as indices for recent growth

RNA/fresh weight has been suggested an index by Pierce et al. (1999), who have found a good correlation with recent growth in captive *Loligo forbesi*. Houlihan et al. (1998) also found RNA/fresh weight to be correlated with growth rate in the octopodid species *E. cirrhosa*. The present study provides further evidence that, in fact, [RNA] increases with higher feeding rates in cephalopod species. Significantly higher [RNA] was found in mantle muscle tissue samples of the HF groups, which corresponded to the higher instantaneous growth these animals displayed (Fig. 1).

RNA/DNA ratios followed a similar pattern, except that significant effects between the two factors, temperature and ration, became evident, which better reflects the groups' attained growth rates than [RNA] did. An increase in the growth-predicting capability of RNA/DNA ratios in comparison to [RNA] also became evident in experiment 2 results: starving time

could explain 89% (17.5 °C) and 78% (22.5 °C) of encountered variability in *G* values and also 51% and 74% of variability in RNA/DNA ratios, while only explaining 26% and 51% of variability in [RNA] (Table 2, Fig. 3).

Buckley et al. (1999), reviewing studies that measured nucleic acid concentrations in marine fish species, concluded that "...when RNA/DNA, RNA/protein, protein/DNA and RNA concentration[s] are correlated with recent growth, RNA/DNA explains the largest part of the observed variability in growth." The present results suggest that this is also the case with cephalopod muscle tissues.

This phenomenon may have at least two reasons: first, DNA concentration may rise, given cell dry weight decreases during starvation, thus amplifying the RNA/DNA signal (Buckley et al., 1999), while on the other hand, RNA/DW ratios would not decline as much. This is the case with experiment 2 results, where at both temperatures, [RNA] in contrast to RNA/DNA ratios did not decline noticeably during the first 2 days of starvation. A slight rise in [DNA] during this time interval explains the decrease in RNA/DNA ratio.

Secondly, the ratio may depend on the degree of hyperplastic vs. hypertrophic growth present in the respective muscle tissue, which may make it necessary to normalize RNA concentration to cell number (dividing by [DNA]; Bulow, 1970), since it cannot be assumed that cells of different size and age are characterized by the same [RNA] at a given nutritional state.

Both experiments of this study demonstrate that higher growth rates at 23 °C/22.5 °C in comparison to 18 °C/17.5 °C could be attained with lower RNA concentrations. Since 80–90% of a cell's RNA is ribosomal (Millward et al., 1973; Westermann and Holt, 1988), it follows that an increase in [RNA] indicates an increase in ribosome concentration and, subsequently, that the translation rates at the ribosomal sites have to be higher at higher temperatures in order to reach higher growth rates at lower or similar [RNA]. This corresponds to results of Goolish et al. (1984), who acclimatized juvenile carp (*Cyprinus carpio*) to different temperatures and compared RNA/DNA ratios from white muscle tissues at a variety of equal growth rates. Proof for varying translational efficiency in a fish species has been supplied by Smith (1981), who found higher [RNA] and higher protein synthesis rates per unit RNA in fed rainbow trout (*Oncorhynchus mykiss*) than in starved ones. A similar pattern could be demonstrated by Houlihan et al. (1990a,b) for the cephalopod *Octopus vulgaris*. These authors calculated RNA activity (expressed as g protein synthesised g^{-1} RNA day^{-1}) in mantle muscle tissue to increase from 6.1 at 0% daily growth to 11.3 at 6% daily growth, but also found an increase in RNA concentration (expressed as unit RNA/unit protein) with growth rate.

Koueta et al. (2000), who maintained groups of 2-week-old *S. officinalis* at three different rations at 19 °C, found significant differences in [RNA] between low and medium ration groups, while between medium and high ration groups, no differences in [RNA] could be detected, despite significantly higher growth rates at the high ration group. Two conclusions can be drawn from their results: (1) more protein is being produced at the high ration level with the same ribosome concentration as in the medium ration group, and (2) there may exist a maximum number of ribosomes that is still compatible with the overall cellular organisation and fast-growing cephalopods may have realised this state. Goolish et al. (1984), in contrast, have observed linear increases in RNA/DNA ratios in fish muscle tissue at various temperatures with growth rate that did not stagnate at the higher growth rates. Cephalopods, as the only invertebrate group that successfully competes in similar ecological niches than fish do (Packard, 1972), rely on a fast generation turnover to escape predatory pressure by fish species (O'Dor and Webber, 1986). They are optimized to reach growth rates comparable to mammals of similar size (Calow, 1987) by displaying very high protein retention rates up to over 90% (compared to 36% for cod, *Gadus morhua*) with higher protein synthesis rates per unit RNA than fish species (Houlihan et al., 1990a,b, 1989). Smith et al. (2000) found RNA translational efficiency in fish fibroblast cells to be associated with increased RNA synthesis and turnover. Assuming high translational efficiencies in *S. officinalis* mantle muscle tissue at high growth rates and further assuming that these elevated efficiencies be allowed by a high RNA turnover rate as shown for the fish cells, stagnating overall concentrations of RNA at higher growth rates could be explained. In our study, only two different

rations could be offered in experiment 1; thus, we could not investigate such an effect in this particular size class of *S. officinalis*, although this would be useful in the evaluation of RNA/DNA ratios as an index for higher growth rates in cephalopods. Given RNA turnover increases with growth rate in favor of increased translation efficiencies to an extent that [RNA] cannot be elevated anymore, RNA/DNA ratios could not distinguish between these high rates of growth. Future research should focus in this direction.

Another problem for the suitability of RNA/DNA ratios as an index for high growth rates is the fact that cephalopods are able to generate new muscle cells throughout their lifecycle (Moltschaniwskyj, 1994). Thus, the predictive capability of RNA/DNA ratios for growth processes may be limited to tissues that mainly perform hypertrophic growth processes, since RNA production during cell multiplication (accompanied by simultaneous DNA replication) might not alter the RNA/DNA ratio. Hyperplastic growth processes might be more easily predicted by measuring activities of enzymes engaged at key positions of nucleic acid metabolism. Koueta et al. (2000) could successfully distinguish between their (abovementioned) medium and high ration groups by determining muscle ATCase activity (the enzyme that controls the first specific step of de novo pyrimidine base synthesis). This indicator of growth would also reflect potential elevated RNA turnover rates with increasing growth rate.

Nonetheless, starving processes are well reflected in the RNA/DNA ratio at both temperatures examined. After an initial period of 2–3 days during which probably only translational activity is being modified according to the new nutritional regime, cellular ribosome concentration is being reduced (Houlihan et al., 1990b; Buckley et al., 1999 for cod *G. morhua* larvae, this study, experiment 2). Thus, a decline in RNA/DNA ratio can be recorded.

4.4. DGI as index for recent growth

DGI proved to be a very reliable indicator of recent growth in juvenile cuttlefish (*S. officinalis*). No biochemical index produced such high relative differences between LF and HF groups at experiment 1 and such marked differences between animals from different starving groups at experiment 2. A role of the digestive gland as a short-term energy reserve in cephalopods has been suggested by various authors (Castro et al., 1992, 1993; Castro and Lee, 1994; Houlihan et al., 1998). Castro et al. (1992) have shown that after 4 days of starvation, 61% of body weight loss of *S. officinalis* specimens could be attributed to digestive gland weight loss, while after >53 days of starvation, a mere 18% of weight loss could be attributed to the digestive gland. Thus, long-term starvation seems to go along with the utilization of muscle protein, while during short-term starvation, significant parts of the metabolic energy are being supplied by the digestive glands' lipids and proteins (Castro et al., 1992; O'Dor et al., 1984 for *O. vulgaris*). Castro et al. (1992) observed their digestive gland index (digestive gland DW/DW rest of the body (excluding the cuttlebone*100) to decrease sharply from 10.5 to 4.9 during the first 4 days and to level off at about 4 during the rest of the starving interval. This corresponds to the exponential decline in DGI (digestive gland FW/animal FW) witnessed in this study at both temperatures during the 6 days of starving trial. The possibility to construct a common regression line of log DGI values vs. starving time from both temperatures tested illustrates the potential this (relatively easy determinable) index may bear to predict the feeding regimen specimens could have recently encountered, given that further animals at different temperatures and from a broader size range were subjected to similar conditions in a larger calibration study.

5. Conclusion

It becomes evident that for certain purposes, the use of biochemical indices for recent growth estimation is advisable, especially if species are very small and digestive glands cannot be dissected within a reasonable time period. Especially for loliginid/ommastrephiid early life stages, RNA/DNA ratios could be useful to estimate losses due to starvation during the transition from an interior food supply (yolk) to exogenous feeding, which represents the most critical time period in the life of a cephalopod. Vidal (2000), working with early life stages of *L. opalescens*, found RNA/DNA ratios to decline with starving time and could identify a "point of no return"

(sensu Blaxter and Hempel, 1963), a starvation period after which animals could not recover after refeeding (5 days in 14-day-old *L. opalescens*). Corresponding RNA/DNA values declined asymptotically with starving time—a phenomenon that has also been found in fish larvae (Clemmesen, 1994 for *Clupea harengus*; Grønkjær et al., 1997 for *G. morhua*). Although minimum RNA/DNA ratios were already reached before the point of no return, it should be possible to derive an estimator for natural mortality of paralarvae in the field from laboratory calibration trials, given that high variances in RNA/DNA ratios within treatment groups encountered (Clarke et al., 1989; see above, Vidal, 2000) are being eliminated with improved nucleic acid assays like the one presented here and that a decline in RNA/DNA ratio with starving time does actually represent a universal phenomenon in cephalopod organisms.

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